Optimization and validation of a rapid method for lipid determination in *Nannochloropsis oculata*

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INTRODUCTION

Microalgae contain high amounts of (neutral) lipids
= promising source for biodiesel and food applications

Lipid content determination

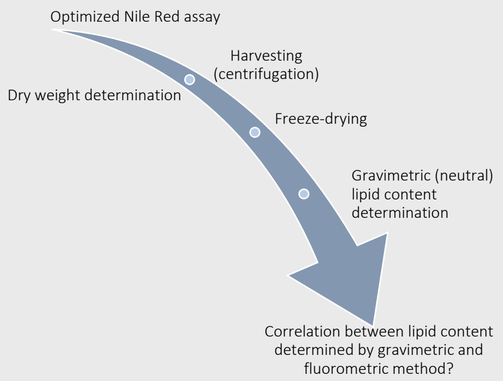
- *Traditionally: gravimetric methods*
  - Advantage: reliable, reproducible
  - Drawbacks: time consuming, labor intensive, high amounts of sample required (not suitable for screening)
- *Alternative: fluorometric method with Nile Red*
  - Advantage: faster
  - Remark: a good validation of this method = correlation between fluorometric and gravimetric method on **independent** samples
    → This has almost not been done!

    Mistakes in other studies:
    - Quantification with external standard curve (e.g. triolein)
      Problem: Nile Red interacts differently with "free" lipid globules than with lipids inside a cell wall (De La Hoz Siegler *et al.*, 2012)
    - Different samples taken from the same batch at different times = not independent!

EXPERIMENTAL SETUP

- **Optimization** of the method
  - Incubation time
  - Measuring temperature
  - Nile Red concentration
  - Addition of glycerol: improves penetration of the dye through the cell wall (Doan *et al.*, 2011)
- **Validation**: variation of
  - Nitrogen concentration in medium
  - Harvesting time

  → Independent samples



- **Total lipid content**: chloroform/methanol extraction according to Ryckebosch *et al.* (2012)
- **Neutral lipid content**: Solid Phase Extraction (SPE) into three lipid fractions: neutral lipids, glycolipids and phospholipids

RESULTS AND DISCUSSION

Emission spectra

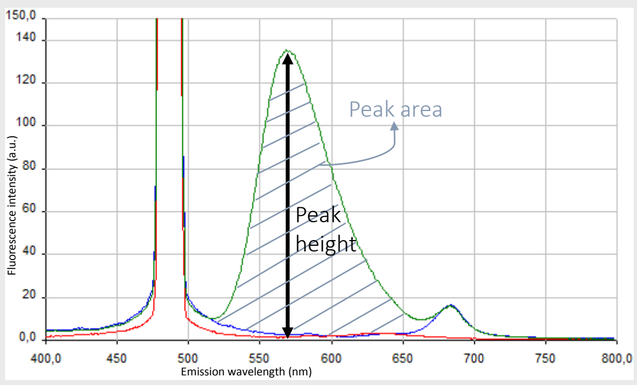


Figure 1: Emission spectra between wavelengths 400 and 800 nm (excitation wavelength = 486 nm)

Relative peak area = area (*N. oculata* with Nile Red) – area (*N. oculata* blank without Nile Red) – area (Nile Red solution)
Relative peak height = height (*N. oculata* with Nile Red) – height (*N. oculata* blank without Nile Red) – height (Nile Red solution)

Optimization

- **Incubation time** (Figure 2)
  Measurement after minimum 90 minutes of incubation at room temperature
- **Measuring temperature** (results not shown)
  At higher measurement temperatures, fluorescence intensity increases (limit = 45˚C), but sample has to adapt to the higher temperature in measuring cell → room temperature has been chosen (25˚C)
- **NR concentration** (Figure 3)
  Fluorescence intensity reaches a maximum at Nile Red concentration of 0,5 mg/mL
- **Glycerol addition** (Figure 4)
  - Addition of glycerol improves fluorescence intensity
  - Maximal fluorescence at 0,25 mL

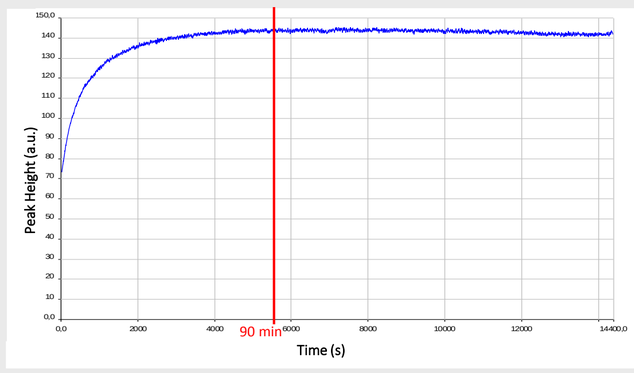


Figure 2: Fluorescence intensity (excitation wavelength 486 nm, emission wavelength 570 nm) in function of time after addition of Nile Red (at room temperature)

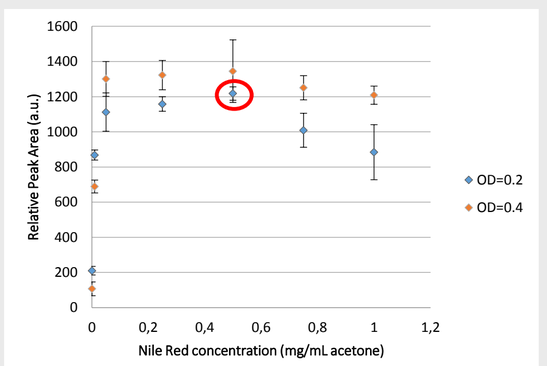


Figure 3: Influence of Nile Red concentration and OD on Nile Red fluorescence intensity (glycerol volume and volume of Nile Red solution remained constant)

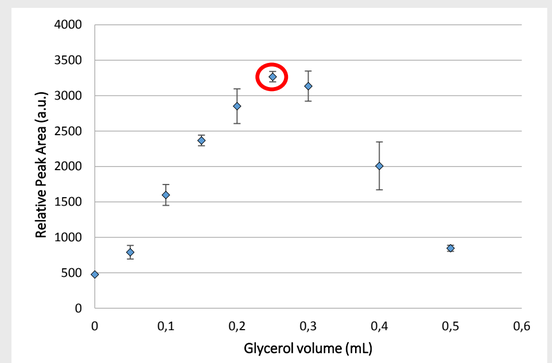


Figure 4: Influence of glycerol volume on fluorescence intensity (volume and concentration of NR solution remained constant)

OPTIMIZED PROTOCOL

- 3 mL of algal suspension of OD_{750}=0,2
- Spectrofluorometric measurement of blank sample (excitation at 486 nm, emission spectrum from 400 to 800 nm, measurement temperature 25˚C)
- Addition of **0,25 mL of glycerol**
- Addition of 10 µL of **Nile Red solution** in acetone (**0,5 mg/mL**)
- Incubation at room temperature for minimum **90 minutes**
- Spectrofluorometric measurement (excitation at 486 nm, emission spectrum from 400 to 800 nm, measurement temperature 25˚C)
- Calculation of both relative peak height and relative peak area (corrected for dry weight)

Validation

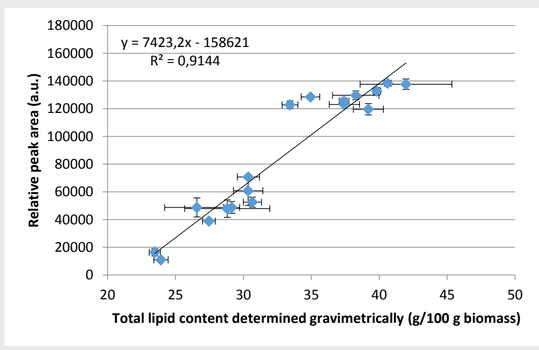


Figure 5: Correlation curve between total lipid content determined by the optimized Nile Red assay and by gravimetry

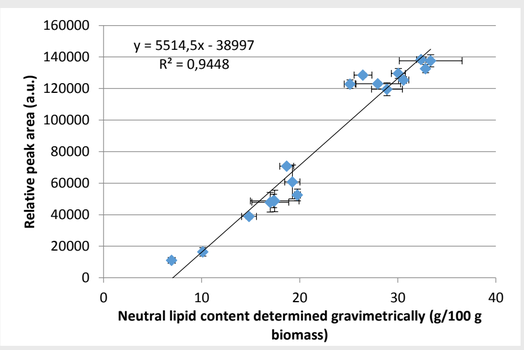


Figure 6: Correlation curve between neutral lipid content determined by the optimized Nile Red assay and by gravimetry

- Good correlation between total and neutral lipid content determined by fluorometric and gravimetric methods
- Slightly better correlation with neutral lipid content (R^2= 0,9448) than with total lipid content (R^2=0,9144), but no big difference because total and neutral lipid content are also correlated in *N. oculata* (R^2= 0,9625)
- Peak height gives the same trends as peak area and comparable correlation coefficients

CONCLUSION

General conclusions

- Penetration of the dye through the cell wall of *N. oculata* was improved by addition of glycerol in an optimal concentration. This leads to an increased fluorescence intensity.
- A good correlation exists between lipid content determined by the optimized Nile Red assay and by gravimetry (R^2=0,9448).
- Gravimetric methods continue to be the most reliable methods for lipid determination, but the Nile Red assay can be used as a screening method (provided that a correlation curve for the used species has been made first!).

Future research

- Optimization and validation for *Isochrysis* is ongoing. The optimized protocol for *N. oculata* can be used, but addition of glycerol has less effect (as *Isochrysis* has no strong cell wall). Validation seems to give another correlation curve.
- Determine if correlation curve is also strain dependent or only species dependent

References
De La Hoz Siegler, H., Ayidzoe, W., Ben-Zvi, A., Burrell, R.E. & McCaffrey, W.C. (2012). Improving the reliability of fluorescence-based neutral lipid content measurements in microalgal cultures. *Algal research*, 1: 176-184.
Doan, T.-T. Y. & Obbard, J.P. (2011). Improved Nile Red staining of *Nannochloropsis* sp. *Journal of Applied Phycology*, 23: 895–901.
Ryckebosch, E., Muylaert, K. & Foubert, I. (2012). Optimization of an analytical procedure for extraction of lipids from microalgae. *Journal of the American Oil Chemists' Society*, 89: 189–198.